DNase-seq protocol for *Drosophila* embryos

DNase I Treatment of Nuclei Isolated from Drosophila Embryos

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This protocol is partly based on a DNase I treatment protocol provided by Peter Sabo from John Stamatoyannopoulos' lab at University of Washington.

Nuclei Prep

Prior to Isolation of Nuclei:

1. prepare stop solution. Add 15ml 10mg/ml RNase A, 9ml 0.5 M spermine, and 15ml 1 M spermidine to 15 ml aliquot of 1XStop Buffer, (12.5 ml needed for each digestion series (2.5 ml reactions))
3. Prepare fresh 1XDNase I Buffer (Dilute 10X DNase I buffer 1:10 with BufferA)
4. Aliquot DNase I Buffer: 50ml conicals, 2.5 ml each (1ml per 1-2x10^7 nuclei).
5. Prewarm Stop Buffer and DNase I Buffer (no DNase I) in 37°C water bath.

Embryo collection

1. Collect embryos from population cages, allow the embryos to develop to desired developmental stage; harvest and dechorinate the embryos.
2. Resuspend in 5 ml cold buffer A (with spermine and spermidine + 0.5 mM DTT, + 1 mM PMSF) per gram of embryos
3. Use motor-driven dounce homogenizer to partially homogenize the embryos (treat 2-3 x at 7000-8000 rpm)
4. Pass the homogenate through Miracloth pre-wetted with cold H_2O.
5. Further homogenize using a dounce homogenizer, pestle B, 5-6 strokes.
6. Add 10 % IGEPAL CA630 drop-wise to final concentration of 0.5%, mix well.
7. Spin 1.5 ml aliquots in microcentrifuge at 3 krpm at 4°C for 3 min.

Nuclei Isolation

1. Re-suspend gently each nuclei pellet in 1 ml of fresh buffer A. Take a small aliquot and set aside for nuclei count.
2. Spin the nuclei down at 3 krpm at 4°C for 3 min.
3. Repeat steps 1-2.
4. Re-suspend the nuclei in each tube into 50 ul, and combine all nuclei.
5. Add proper amount of nuclei (5-7x10^7 nuclei per 2.5 ml reaction) to a set of 50 ml Falcon tubes for DNase I concentration series.

Nuclei Count

1. dilute nuclei 1:10, Count nuclei on the hemacytometer while pelleting nuclei.(count can be done in buffer A)

DNase I treatment

- Use Roche DNase I at 10,20,40,60 units/ml (non-Mg++ buffer)

1. Equilibrate the Stop Buffer and a set of 50 ml Falcon tubes containing 2.5 ml aliquots of 1x DNase I buffer to 37°C, by incubating in a 37°C water bath for 30 minutes.
2. Add 2.5, 5, 10, 15 ul (10 units/ml) DNase I enzymes to each of the equilibrated tubes. Mix thoroughly by pipetting.
3. Place tubes with nuclei pellets in 37°C water bath and allow temperature to equilibrate for 1 minute at 37°C.
4. Re-suspend nuclei pellet with 1x DNase I buffer plus enzyme, pipette several times gently to ensure homogenous suspension.
5. Incubate for 3 minutes at 37°C.
6. Add equal volume of stop buffer to each reaction tube, mix by inverting several times, transfer tubes to 55°C water bath.
7. Allow digestion tubes to incubate at 55°C for 15 minutes then add 2.5 ml proteinase K per ml DNA solution. (Invitrogen 10 mg/ml).
8. Allow digestion with Proteinase K to continue overnight (min. 16 hr.) at 55°C.
9. Store at 4°C.

Stock Buffers and Solution Preparation

Unless otherwise noted, all buffers and reagents should be filtered (0.22μm) and pre-chilled to 4°C (on ice) before use.

Buffer A (50 ml)

Final Concentration	*Stock conc.*	*Amount to add*
15 mM Tris-Cl, pH 8.0	1 M	0.750 mL
15 mM NaCl	5 M	0.15 mL
60 mM KCl	3 M	1 mL
1 mM EDTA, pH 8.0	0.5 M	0.1 mL
0.5 mM EGTA, pH 8.0	0.25 M	0.1 mL
0.5 mM Spermidine	1 M	25 μl
H_2O, sterile		47.875ml

Buffer A + spermine

50 ml buffer A+15 μl 0.5 M spermine(final 0.15 mM)

DNase I 1X Digestion Buffer (25 mL)

Final Concentration	*Stock conc.*	*Amount to add*
6 mM CaCl2	1 M	0.15 mL
75 mM NaCl	5 M	0.375 mL
Buffer A		24.475 ml

Stop Buffer (50 ml)

Final Concentration	*Stock conc.*	*Amount to add*
50 mM Tris-Cl, pH 8.0	1 M	2.5 mL
100 mM NaCl	5 M	1 mL
0.10 % SDS	20%	0.25 mL
100 mM EDTA, pH 8.0	0.5 M	10 mL
H_2O, sterile		36.25 ml

Dispense into 50-ml aliquots store at 4°C. (SDS will precipitate at 4°C but will go back into solution upon heating to 55°C)

On day of use add the following:
1) 50 ml 10 mg/ml RNase A (final concentration 10 ug/ml)
2) 100 ml 0.5 M Spermidine (final concentration 1 mM)
3) 30 ml 0.5 M Spermine (final concentration 0.3 mM)

10% IGEPAL CA630 Stock (per 100 ml):

Final Concentration	*Stock conc.*	*Amount to add*
10% IGEPAL CA630	100% IGEPAL CA630	10 mL
H_2O, sterile		90 ml

*100% IGEPAL CA630 is extremely viscous; be careful to accurately withdraw 10 ml. 10% IGEPAL CA630 should be warmed in a 55°C waterbath for 30 minutes prior to use to ensure proper dissolution. Note in our experiments we used a stock of the original NP-40 detergent, which is no longer available, rather than IGEPAL CA630. IGEPAL CA630 is the correct substitute for the original NP40 detergent. Do not use any currently available chemicals called NP40 as these are not the same thing.

Store at 4°C

Cleaning digested DNA samples prior to running on sucrose gradients

Peter Sabo, University of Washington

PPE required: Lab coats, Safety glasses and nitrile gloves should be worn while performing this protocol. Pipetting of phenol and chloroform should be done in the fume hood.

Ordering Information

Item.	Catalog No	Manufacturer
Phenol/chloroform	77617	Sigma
Chloroform	C2432	Sigma
Phase Loc tubes Heavy, 15 ml	2302850	5 Prime
Phase Loc tubes Heavy, 50 ml	2302870	5 Prime

Materials List

15 ml and 50 ml polypropylene Conical Tubes
Phase Loc heavy 15/50 ml tubes
Phenol/Chloroform isoamy alcohol
Chloroform
Glass pipets

1. Place sample in 55°C water bath for 10 minutes.

2. Transfer sample to PhaseLoc tubes. (Transfer should be done by pouring to minimize shearing)

   Transfer sample to 15 ml or 50 ml PhaseLoc tube by pouring from original tube. Do not pipet samples to transfer.

   If sample is less than 8 ml transfer to a 15 ml phaseloc.
   If sample is between 8 and 35 ml it should be transferred to a 50 ml phaseloc.
   If sample is larger than 35 ml's should be split between 2-50 ml phaseloc tubes.

3. Add 1/3 volume, up to 10 mls, Phenol/chloroform to sample using glass pipets.
4. Secure caps and verify they do not leak.
5. Secure tubes onto LabQuake Rotisserie.
6. Turn on and rotate samples for 10 minutes.
7. Turn off, remove tubes and transfer to RT Centrifuge.
8. Centrifuge 5 minutes at 1500 xg, 25°C.
9. If tube does not allow addition of 1/3 volume chloroform transfer sample to new phase lock tube by pouring into new tube.
10. Add 1/3 volume chloroform using glass pipet.

11. Secure caps and verify they do not leak.
12. Secure tubes onto LabQuake Rotisserie.
13. Turn on and rotate samples for 10 minutes.
14. Turn off, remove tubes and transfer to 25°C Centrifuge.
15. Centrifuge 5 minutes at 1500 xg, 25°C.
16. Check to make sure there is solid separation of organic and aqueous
17. Pour off sample into clean tube.
18. Store sample at 4°C until needed.
19. Dispose of phenol chloroform containing tubes and contaminated tips in chemical waste stream.

Sucrose Fractionation

Peter Sabo, University of Washington

Description: method for the separation of DNA fragments based on mobility within 9% sucrose cushion. Used to isolate small fragments suitable for sequencing.

PPE requirements: Laboratory coats and nitrile gloves should be worn while performing isolations.

Ordering Information

Item.	Catalog No	Manufacturer
14 x 89 mm Polyclear Tubes	7030	Seton Scientific
Sucrose Ultracentrifuge grade NaCl	BP220-1	Fisher Scientific

Equipment / Materials List

Beckman ultracentrifuge swing bucket rotor SW 41 or equivalent
Beckma XL90 Ultracentrifuge or equivalent
14 x 89 polyclear tubes or equivalent
1 ml wide bore pipet tips

Sucrose cushion (day1)

Start setup first thing in morning.

Sample to be run should have been previously cleaned using Phenol ChCl3 protocol.

1. Add 10 ml 9% sucrose solution to 6 ea 14 x 89 mm Polyclear Tubes. (Seton Scientific 7030)
2. Place sample in 55°C water Bath for 10 Minutes.
3. Gently load 1-2 ml's of sample using wide bore pipet onto surface of sucrose.
4. Weigh tubes to make sure they are balanced to within 0.05 grams of each other.
5. Load tubes into SW41 buckets and load into SW41 rotor in Ultracentrifuge.
6. Run the following program:

Rotor	SW41
Speed	25,000 rpm
Time	24 hours
Acceleration profile:	4
Deceleration profile:	**No Brake**.
Temp	20°C

Pull off fractions from sucrose cushion (day 2)

1. After centrifuge has stopped and vacuum released carefully remove tubes and place in rack.
2. From top down, carefully pipet off 0.5 ml fractions into 96 well 1.1ml plate.

   Take 10-16 fractions.
3. load 8 ul on 2.0% TAE agarose gel
4. Load 100 bp DNA ladder.
5. Run 50 minutes 90 volts in mini gel format.
6. Post stain sybr green for 1 hr.
7. Image gel using Typhoon 9200 or equivalent laser scanner. Due to the small amount of DNA present a laser scanner or instrument with similar sensitivity is recommended as you may not see anything with a camera based system.
8. Identify and pool fractions (in 15ml conical tube) that are below 700-500 bp. (usually fractions 1-9)

Cleanup DNA fragments (day2)

1. Add 3 volumes qiagen Buffer QG to pooled sucrose fractions.
2. Add 0.7 volumes isopropanol and mix by inverting.
3. Place 1 qiagen mini PCR column onto vacuum manifold and apply vacuum.
4. Load sample on to column and vacuum through.
5. Load 600 ml's Buffer PE onto column and vacuum through.
6. Transfer PCR column to spin collection tube supplied with column and spin 3 minutes at 9,000 xg in microfuge
7. Transfer spin column to 1.5 ml low bind Microfuge tube.
8. Add 400 ul Elution buffer and allow sample to sit 5 minutes.
9. Spin 9000 xg for 2 minutes in microfuge.
10. Take 5 ul's of flow through and set aside for qc gel.
11. Add 1 ul glycogen
12. Add $1/0^{th}$ volume of 3M Sodium Acetate
13. Add 2.0 volumes of 100% Ethanol.
14. Mix and place in -20°C freezer for 2hrs to overnight.
15. Centrifuge 14,000 xg for 10 minutes
16. A glycogen/DNA pellet may be visible at bottom of tube.
17. Aspirate off solution carefully avoiding pellet.
18. Add 500 ml 70% Ethanol

19. Spin 5 minutes 14,000 xg.
20. A glycogen/DNA pellet should be visible at bottom of tube.
21. Aspirate off solution carefully avoiding pellet.
22. Dry in speedvac for minutes.
23. Add 10 ul 10 mM Tris to sample and either proceed with library construction or store at -20°C.

9% Sucrose Solution: 1 Liter.

90 grams Sucrose Ultra Centrifuge grade.	9%
54.8 Grams NaCl.	1 M
Bring up to 970 ml with Sterile DI H2O	
20.0 ml 1M Tris pH 8.0	20 mM
5.0 ml 0.5 M EDTA	2.5 mM
Filter Sterilize 0.2 um.	
Store at 4°C	

Construction of digital DNase I libraries for sequencing

Taken from Hesselberth et al (2009) Nature Methods 6, 283.

Digital DNase I libraries were constructed according to Illumina's genomic prep kit protocol with minor modifications. 50 ng of purified DNase double-hit fragments (100-500 bp) were subjected to end repair by combining fractionated DNA, 1X T4 DNA ligase buffer (containing 0.1 mM ATP), 0.4 mM dNTP mix, 15 U T4 DNA polymerase, 5U DNA polymerase (Klenow, large fragment) and 50 U T4 polynucleotide kinase (all from New England Biolabs, Ipswich, MA). The reaction mixture was incubated at 20 °C for 30 minutes and purified using a MinElute micro spin column (Qiagen). Following endrepair, non-templated adenines were added to the 3' ends of purified fragments with 13 mM dATP and 5 U Klenow fragment (3'- 5' exo-) (NEB) and incubated at 37 °C for 30 minutes. After column purification, Illumina adapters were ligated to the ends of DNA fragments in a 50uL reaction by combining 17.5 µL 'A'-tailed fragments, 25 ul 2X Quick DNA ligase buffer (NEB), 2.5 µL adapter oligo mix (Illumina, Hayward, CA) and 5uL Quick DNA ligase (NEB) and incubating the mixture for 15 minutes at room temperature. Ligations were purified with MinElute columns (Qiagen).

Adapter-ligated DNA fragments were enriched by amplification. 50 uL reactions consisting of 5 ul adapter-ligated DNA, 25 ul 2X Phusion High-Fidelity PCR Master Mix (NEB), 0.5 ul each of primer 1.1 and 2.1 (Illumina, Hayward, CA) and 19 uL nucleasefree water were assembled and cycled with the following thermal profile: 98 °C for 30 min., followed by 16 cycles of 98 °C for 10 min, 65 °C for 30 min. and 72 °C for 30 minutes with a final extension at 72 °C for 5 minutes. Amplified libraries were purified with a MinElute micro spin column (Qiagen) and quantified using PicoGreen stain (Invitrogen) and a NanoDrop 3300 fluorospectrometer (Thermo Scientific, Waltham, MA).

DDHF libraries were the sequenced by the University of Washington High-Throughput Genomics Unit to produce 27 bp reads according to standard sequencing-by-synthesis methodology on an Illumina Genome Analyzer (GA1).